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{Exhibit 65}

So, et al., "Characterization of an Escherichia coli Plasmid Encoding for the Synthesis of Heat-Labile Toxin: Molecular Cloning of the Toxin Determinant", Infection and Immunity, 21: 405-11(1978)

Characterization of an *Escherichia coli* Plasmid Encoding for Synthesis of Heat-Labile Toxin: Molecular Cloning of the Toxin Determinant

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P307 is a plasmid isolated from a strain of *Escherichia coli* that was responsible for an outbreak of diarrheal disease in piglets. This 60×10^6 -dalton plasmid was subsequently shown to encode for the synthesis of a heat-labile toxin (LT). Using recombinant DNA technology, we isolated a 7.8×10^6 -dalton DNA fragment that contains the LT gene(s). This fragment was generated using an EcoRI partial digestion of P307 DNA, and the fragment was joined to a small multicopy plasmid, RSF2124. *E. coli* strains harboring the chimeric plasmid produced greater amounts of LT than did the same strains containing P307. The LT genes were also isolated on a 5.8×10^6 -dalton DNA fragment made by BamHI digestion, and we identified an EcoRI recognition sequence that is located in a position essential for LT synthesis.

Certain strains of *Escherichia coli* cause a disease which resembles cholera and is associated with one or more enterotoxins. Two general types of *E. coli* enterotoxins are now recognized (28). One type of toxin, ST, is a nonimmunogenic, heat-stable polypeptide variously estimated at 1,000 to 10,000 daltons (24). The mode of ST action is still unknown, and the activity of the toxin is assayed using a suckling mouse model (5).

The other type of toxin is an immunogenic protein which loses its activity after heating at 60°C for 10 min (13, 15). It is designated LT for heat-labile toxin and its molecular weight has been variously estimated to be from 24,000 to over 100,000 (7, 24). LT shares partial immunogenic identity with cholera toxin (14, 32) and, like the latter, exerts its effect by stimulating adenyl cyclase activity in epithelial cells of the small bowel (6, 12). Tissue culture assays with either Chinese hamster ovary (CHO) cells (12) or Y-1 mouse adrenal cells (6) are now widely employed to detect LT activity.

Enterotoxigenic *E. coli* have been isolated from calves, piglets, lambs, and humans (4, 8, 10, 13, 26, 31). In most cases, the genetic information for the synthesis of both ST and LT has proved to be plasmid mediated (16, 26, 27, 30, 31). One recent report indicates that LT synthesis is also occasionally linked to phage conversion (36). In an earlier paper, we reported the successful use of DNA recombinant technology for the isolation

of a DNA fragment containing the gene(s) for ST synthesis (35). In this paper we report the application of similar molecular techniques to the isolation of the genetic sequences encoding LT.

MATERIALS AND METHODS

Bacterial strains. All strains used are derivatives of *E. coli* K-12. C600 is *thr*⁻ *leu*⁻ *B1*⁻ *lac*⁻ *supE*. 1485 is *thy*⁻ *B1*⁻ *lac*⁺ *Nal*^r. 711 (P307) is *his*⁻ *pro*⁻ *phe*⁻ *trp*⁻ *lac*⁻ *Nal*^r and contains the Ent ST + LT plasmid P307 (13, 16, 32). Strain 1485 (F⁺) was kindly provided by E. Ohtsubo. Plasmid RSF2124 has been described previously (34).

Isolation of plasmid DNA. Strains containing the plasmids to be isolated were grown in brain heart infusion broth (Difco) overnight. Cells were harvested by centrifugation and washed once with sterile buffered saline or TE buffer [10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.4, 10 mM ethylenediaminetetraacetic acid (EDTA)]. The cells were resuspended in 14 ml of 25% (wt/vol) sucrose dissolved in TES buffer (30 mM Tris, pH 8, 5 mM EDTA, 50 mM NaCl), 0.1 ml of lysozyme (10 mg/ml) was added, and the solution was iced for 15 min. Two milliliters of 0.5 M EDTA was added. After 10 min, 11 ml of Triton lytic mix (0.1% [wt/vol] Triton X-100, 50 mM EDTA, 50 mM Tris, pH 8) was added. Cells were iced for an additional 15 min or until visible clearing of the solution. The lysate was centrifuged at 18,000 rpm for 25 min at 4°C in a Sorvall RC-2B centrifuge. TES buffer was added to the supernatant to make a total volume of 22 ml, and 1 ml of ethidium bromide (10 mg/ml) was added. The density of the solution was adjusted

to 1.3925 ± 0.001 with CsCl. The solution was centrifuged to equilibrium in a Ti60 rotor (Beckman) in a Beckman L3-50 ultracentrifuge (ca. 44 h at 40,000 rpm at 15°C). The plasmid band was visualized with an UV light source and was either withdrawn with an 18-gauge needle attached to a disposable plastic syringe or collected by puncturing the polyallomer tube from the bottom and collecting the plasmid fraction by dripping. The ethidium bromide was extracted with CsCl and water-saturated isopropanol. The DNA sample was dialyzed against 2,000 volumes of 10 mM Tris (pH 8) and 0.5 mM EDTA.

Restriction enzyme reactions. EcoRI and BamHI restriction enzymes were obtained from Miles Laboratories, and complete digestion conditions were as specified by the supplier. Partial digests with EcoRI were done at 0°C for 1 min using various dilutions of the enzyme. Vertical gels of 0.7% agarose were run according to the method described by Greene et al. (11), and 20-cm horizontal agarose gels were run at 3 V/cm in a Lucite apparatus of our own design. Gels were stained in a 25-μg/ml solution of ethidium bromide, and DNA bands were visualized with the aid of a short wave UV light box obtained from Ultra-Violet Products, Inc.

Ligation conditions. *E. coli* DNA ligase was a gift of H. W. Boyer. Ligations were carried out in 0.1 M Tris (pH 8), 0.1 M MgCl₂, 10 mM EDTA, 0.5 mg of bovine serum albumin per ml, and 0.1 mM nicotinamide adenine dinucleotide at a total DNA concentration of ca. 500 μg/ml, with a ratio of cloning vehicle to P307 DNA of 1.5:1.

Toxin assays. LT was assayed by using either CHO cells according to the method of Guerrant et al. (12) or Y-1 mouse adrenal cells as described by Donta et al. (6).

Plasmid copy numbers. Experiments were performed as described by Clewell and Helinski (2). Colicin production was detected by the method of Ozeki (22).

Guidelines employed for recombinant DNA experiments. The experiments reported here were performed using P2-EKI conditions as specified in the *Guidelines for Recombinant DNA Technology*, published by the National Institutes of Health. The potential hazards of working with *E. coli* K-12 carrying recombinant DNA which includes determinants of bacterial pathogenicity have been described in considerable detail elsewhere (21, 35).

RESULTS

Analysis of P307 DNA. The Ent ST + LT plasmid P307 was isolated from an enteropathogenic *E. coli* strain which caused an outbreak of diarrheal disease in piglets (15). P307 is ca. 60×10^6 daltons in mass and has a 0.50-mol fraction guanine plus cytosine content (16). It belongs to the F1 incompatibility complex (25, 33), and its nucleic acid sequence homologies with other Ent and with R plasmids have also been studied (33). We chose P307 for our molecular cloning experiments because it was the best characterized Ent ST + LT plasmid available. The LT elaborated

by cells carrying P307 has been the subject of intensive study and was known to give a strongly positive tissue culture reaction with both CHO and Y-1 cells.

Cleavage of P307 DNA with EcoRI restriction endonuclease yielded 13 fragments. Figure 1C

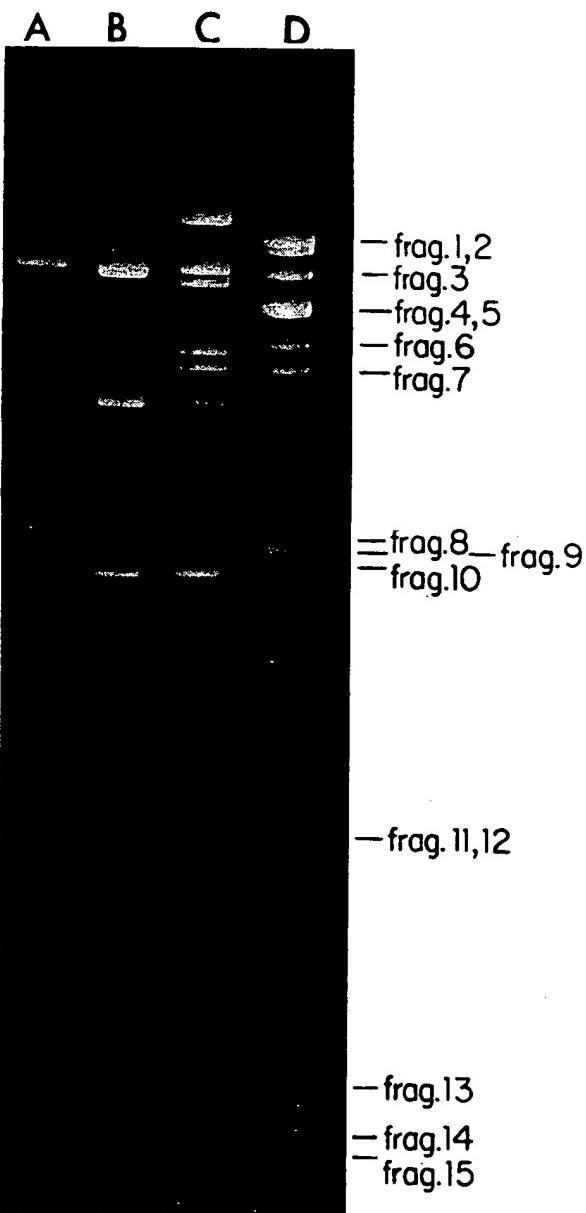


FIG. 1. Migration in a horizontal 0.7% agarose gel of EcoRI-cleaved (A) RSF2124, (B) ESF3003, (C) P307, and (D) F plasmid DNA. Masses of F DNA fragments: 8.95×10^6 , 8.81×10^6 , 7.61×10^6 , 6.10×10^6 , 5.96×10^6 , 5.10×10^6 , 4.84×10^6 , 3.05×10^6 , 2.92×10^6 , 2.85×10^6 , 1.55×10^6 , 1.51×10^6 , 0.93×10^6 , 0.86×10^6 , and 0.80×10^6 daltons. Masses of P307 DNA fragments: 10.3×10^6 , 8.1×10^6 , 7.1×10^6 , 5.2×10^6 , 4.9×10^6 , 4.4×10^6 , 3.1×10^6 , 3.0×10^6 , 2.9×10^6 , 2.7×10^6 (a doublet), 2.6×10^6 , and 0.7×10^6 daltons. Electrophoresis was carried out at 3 V/cm for 20 h (note: F fragments 13, 14, and 15 and P307 fragment 13 cannot be seen in this reproduction).

shows the P307 DNA. 0.7% agarose was used as a determinative P307 fragment 8.1×10^6 , 7.1×10^6 , and 0.7×10^6 average of the sum of reasonable a daltons obtained sedimentation (16). The weight of 2.7 of two distinct similar mole under these smallest Eco 1. P307 fragments 3. digested F pl 14 are contiguous of the transfr relationship s nly in their scope observ agree with t some relation cation region Cloning t RSF2124 (34) our initial re EcoRI digest yielded no L' that a struct necessary for EcoRI recogn real, the pres vide an impor the LT gene ments. Therefor reaction conc P307 DNA pi tigu us EcoR tially digested amounts of a been diluted t zymatic activ EcoRI needed completion in of the reactio sis in a 0.7% ag the preparati ments up to 30 to EcoRI-clea DNA was sec

he subject of
ive a strongly
th both CHO

RI restriction
ts. Figure 1C

sg.1,2
sg.3
sg.4,5
sg.6
sg.7

sg.8—frag.9
sg.10

sg.11,12

sg.13
sg.14
sg.15

7% agarose gel
ESF3003, (C)
ises of F DNA
 1×10^6 , 6.10×10^6 , 3.05×10^6 , 2.92×10^6 , 0.93×10^6 , 0.86×10^6 daltons.
V/cm for 20 h
P307 fragment

shows the fragment pattern of *EcoRI*-digested P307 DNA after electrophoresis in a horizontal 0.7% agarose gel. *EcoRI*-cleaved F plasmid DNA was used as a standard for molecular-weight determinations. The molecular weights of the P307 fragments were estimated to be 10.3×10^6 , 8.1×10^6 , 7.1×10^6 , 5.2×10^6 , 4.9×10^6 , 4.4×10^6 , 3.1×10^6 , 3.0×10^6 , 2.9×10^6 , 2.7×10^6 , 2.6×10^6 , and 0.7×10^6 daltons. Each of these values is an average of three separate determinations, and the sum of these values is 57.7×10^6 daltons, in reasonable agreement with the value of 60×10^6 daltons obtained from neutral sucrose gradient sedimentation of supercoiled P307 plasmid DNA (16). The single DNA band at a molecular weight of 2.7×10^6 daltons is actually composed of two distinct *EcoRI* fragments that have very similar molecular weights and are not resolved under these electrophoretic conditions. The smallest *EcoRI* fragment cannot be seen in Fig. 1. P307 fragments 2, 5, and 13 comigrated with fragments 3, 7, and 14, respectively, of *EcoRI*-digested F plasmid DNA. F fragments 3, 7, and 14 are contiguous with each other and form part of the transfer genes of the plasmid (27). This relationship suggests that F and P307 are related only in their transfer regions. Electron microscope observations of F/P307 heteroduplexes agree with this conclusion as well as showing some relationship between the essential replication regions of F and P307 (23, 25).

Cloning the LT gene(s). The R plasmid RSF2124 (34) was used as the cloning vehicle in our initial recombinant experiments. Complete *EcoRI* digests of P307 DNA ligated to RSF2124 yielded no LT⁺ clones. This finding suggested that a structural gene or controlling element necessary for LT biosynthesis contained an *EcoRI* recognition site. If this observation were real, the presence of this *EcoRI* site would provide an important physical means of identifying the LT gene(s) in subsequent cloning experiments. Therefore, we decided to alter the *EcoRI* reaction conditions in order to generate Ent P307 DNA pieces containing two or more contiguous *EcoRI* fragments. P307 DNA was partially digested for 1 min at 0°C with varying amounts of an *EcoRI* preparation which had been diluted to 1/50 of the original activity (enzymatic activity is defined as the amount of *EcoRI* needed to digest 1 µg of lambda DNA to completion in 30 min at 37°C). Samples of each of the reactions were subjected to electrophoresis in a 0.7% agarose gel to monitor cleavage, and the preparations which contained DNA fragments up to 30×10^6 daltons in mass were ligated to *EcoRI*-cleaved RSF2124 DNA. The ligated DNA was sedimented in a 5 to 20% neutral

sucrose gradient, and fractions collected from the gradient were used to transform *E. coli* K-12 strain C600 (3). Transformants were selected for resistance to 100 µg of ampicillin (Calbiochem) per ml, and 50 clones from each of 13 sucrose fractions were tested for colicin (Col) production. Those clones which were Col⁻ were then tested in CHO cells for LT synthesis. Three clones from one fraction (fraction 9) gave positive LT tests with both CHO cells and with Y-1 cells. These ampicillin-resistant, Ap^r, Col⁻LT⁺ clones were designated as ESF3003, ESF3004, and ESF3005. Plasmid DNA was isolated from each strain and digested with *EcoRI*, and the banding patterns were examined in 0.7% agarose gels. Figure 1B shows the *EcoRI* digest of ESF3003. Four bands are present—one that comigrates with linear RSF2124 DNA (Fig. 1A) and three that comigrate with P307 band 6 (4.4×10^6 daltons), band 10 or 11 (10 and 11 have very similar molecular weights and migrate as a single band of 2.7×10^6 daltons), and band 13 (0.7×10^6 daltons), respectively. The molecular weight of this hybrid plasmid is ca. 15.1×10^6 .

As mentioned above, complete *EcoRI* digests of P307 ligated to RSF2124 gave no LT⁺ clones. To confirm the hypothesis that there was an *EcoRI* site in a DNA sequence essential for expression of LT synthesis, we digested ESF3003 to completion with *EcoRI* and ligated the fragments to RSF2124. The ligated DNA was used to transform C600 as described above. *EcoRI* digest patterns of plasmid DNA from seven Ap^r Col⁻ clones were examined. There was only one P307 DNA fragment in each of the seven strains. *EcoRI* digest patterns of two such strains are shown in Fig. 2. Clones containing any one of the three P307 fragments were tested in both CHO and Y-1 cells, and all proved to be LT⁻. In another experiment, an *EcoRI* partial digest of ESF3003 eliminated the 0.7×10^6 -dalton fragment. These hybrid plasmids contained only the 4.4×10^6 - and 2.7×10^6 -dalton fragments and were LT⁺ (data not shown).

E. coli 711 was transformed with ESF3003 plasmid DNA to test for amplification of LT synthesis (the original Ent plasmid P307 was originally transferred from its wild-type *E. coli* host to 711, so the latter was chosen for the transformation to provide a uniform genetic background). Strains 711 (P307), 711 (ESF3003), and 711 (RSF2124) were grown in PF medium (5), and the broth supernatant was sterilized by passage through a membrane HA 0.45 µm filter (Millipore Corp.). Dilutions of the supernatant were assayed in CHO cells for LT activity. The data in Table 1 show that ESF3003 supernatants contained measurable LT activity beyond a

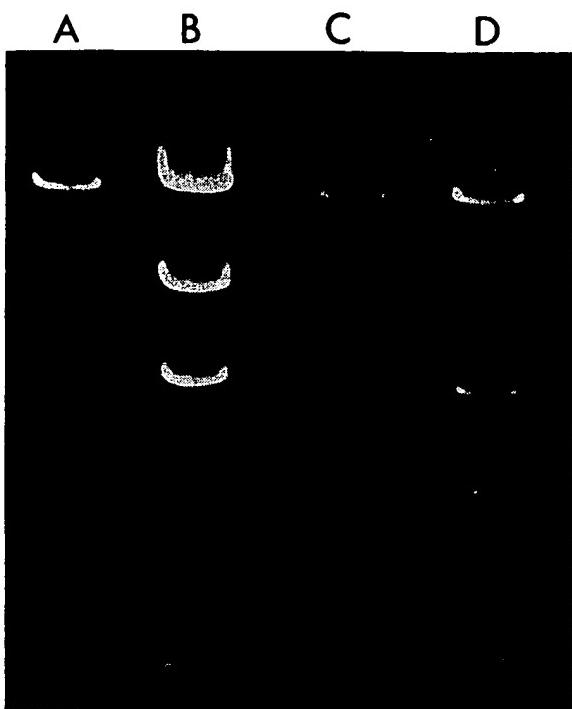


FIG. 2. Migration in a vertical 0.7% agarose gel of EcoRI-cleaved (A) RSF2124, (B) ESF3003, and (C) and (D) plasmid DNAs from a cloning experiment containing RSF2124 and one of the three EcoRI fragments from ESF3003 of P307 origin. Electrophoresis was carried out at 120 V for 2.3 h.

TABLE 1. Effect of dilution of 711 (ESF3003) supernatants on the toxicity to CHO cells^a

Dilution of supernatant	% Cells elongated		
	711 (RSF2124)	711 (P307)	711 (ESF3003)
1/10	<3	60	62
1/40	<3	45	64
1/100	<3	28	65
1/300	<3	3	43
1/500	<3	2	40
1/700	<3	3	25
1/900	<3		13

^a Each value represents data from two to three experiments.

1:700 dilution. In contrast, the 711 (P307) supernatant was devoid of LT activity when diluted beyond 1:100. These should not be taken as absolute figures since this method for determining toxin activity is semiquantitative at best. Nevertheless, 711 (ESF3003) appears to be a more potent LT producer than the original wild-type 711 (P307) strain. Indeed, copy number experiments indicate that the ESF3003 plasmid exists in the cell at an average of 13 to 15 copies per chromosome equivalent, while P307 exists at an average of 0.5 to 1 copy per chromosome equivalent (data not shown).

Recloning the LT gene(s). We attempted to reduce the size of the cloned LT DNA fragment that was made by EcoRI partial digestion by recloning using the restriction enzyme BamHI. This enzyme cuts ESF3003 into three fragments of molecular weights 5.8×10^6 , 4.8×10^6 , and 4.5×10^6 daltons (Fig. 3). In this cloning experiment, the cloning vehicle pBR322 was used. This small, multicopied plasmid carries the genes for Ap^r and tetracycline resistance (Tc^r) and has a single BamHI cleavage site (1). Insertion of a DNA fragment into the BamHI site of pBR322 inactivates the Tc^r gene. Therefore, clones with an Ap^r tetracycline-sensitive (Tc^s) phenotype would be expected to contain an inserted DNA fragment. ESF3003 and pBR322 were cleaved with BamHI, the resultant fragments were mixed together in the presence of polynucleotide ligase, and, after incubation, transformed into *E. coli* C600. Ap^r Tc^s clones were tested for LT

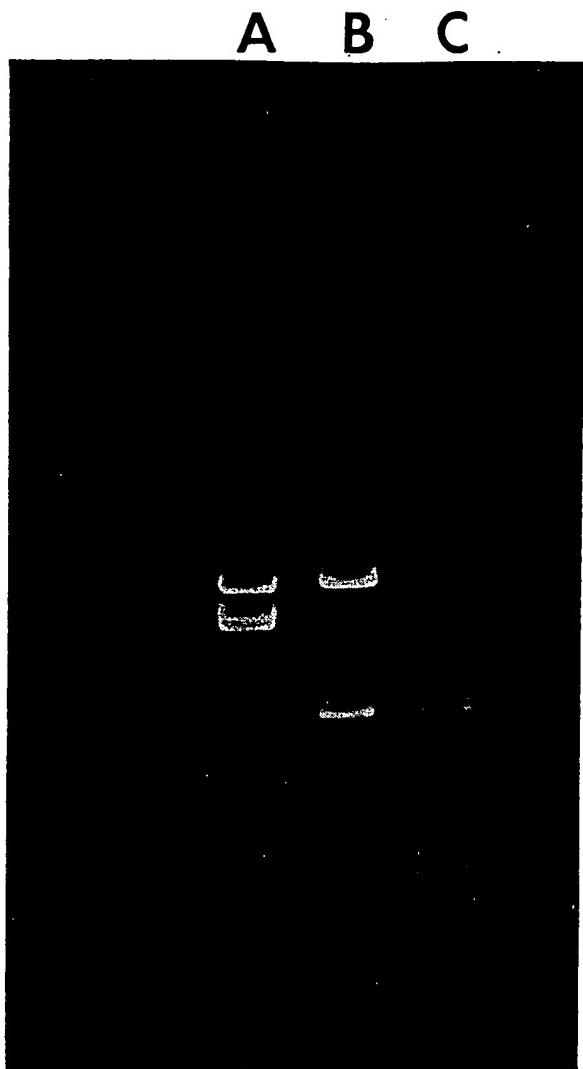


FIG. 3. Migration in a vertical 0.7% agarose gel of BamHI-cleaved (A) ESF3003, (B) EWD030, and (C) pBR322.

FIG. 4. Migration in a vertical 0.7% agarose gel of

BamHI-cleaved (A,

attempted to cleave the plasmid with the enzyme *Bam*HI. Three fragments were obtained, one being 5.8×10^6 , and the other two being 4.4×10^6 and 2.7×10^6 daltons in size. This experiment was repeated with P307 and the same results were obtained. This suggested that the cloned fragment in EWD030 was derived entirely from P307, and this conjecture was verified by the double digestion of EWD030 with the enzymes *Bam*HI and *Eco*RI (data not shown). Figure 5 presents a schematic representation of EWD030 with the *Bam*HI and *Eco*RI recognition sites mapped. EWD030 does not contain the 0.7×10^6 -dalton fragment from the initial *Eco*RI cloning experiments. Also, the 4.4×10^6 - and the 2.7×10^6 -dalton fragments from the original *Eco*RI cloning have been reduced in size to 3.8×10^6 and 1.8×10^6 daltons in mass, respectively.

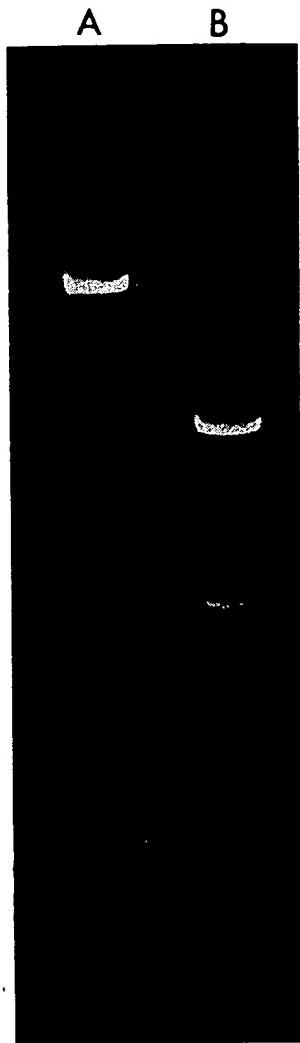


FIG. 4. Migration in a vertical 0.7% agarose gel of *Bam*HI-cleaved (A) P307 and (B) EWD030.

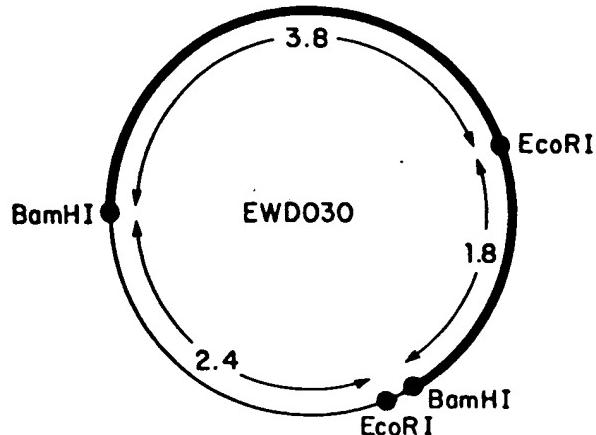


FIG. 5. Schematic diagram of the restriction endonuclease cleavage map of EWD030. The heavy line represents the 5.8×10^6 -dalton *Bam*HI fragment cloned from ESF3003 into *pBR*322. The light line represents the cloning vehicle *pBR*322, which is 2.6×10^6 daltons in mass (l). Intramolecular distances between restriction sites are expressed in daltons $\times 10^6$ of DNA. These distances were obtained by double digestion of EWD030 with the enzymes *Bam*HI and *Eco*RI. The mass of the hybrid plasmid as estimated by agarose gel electrophoresis is 8.3×10^6 daltons.

DISCUSSION

The majority of Ent plasmids isolated are cryptic in the phenotypic sense, since they do not encode a readily identifiable or assayable characteristic. As noted previously, animal models or tissue culture assays are necessary for detecting the enterotoxins encoded by these plasmids. Such assay systems do not lend themselves to large-scale genetic or molecular analysis of the Ent plasmids or their enterotoxins. In addition, most Ent plasmids occur within the bacterial cells as single copies, unlike the small drug resistance plasmids which may exist in as many as 30 copies per cell (9, 17-20). Thus, the physical isolation of both the Ent plasmids and their enterotoxic products remains a difficult task.

Using DNA recombinant techniques, we have transferred the gene(s) for LT synthesis from a relatively large 60×10^6 -dalton conjugative plasmid to several smaller, well-defined nonconjugative plasmids. The 8.4×10^6 -dalton recombinant plasmid, EWD030, is only 1/7 the size of the original P307 parent. If the cloning vehicle is discounted, the *Bam*HI fragment containing the LT gene(s) is only 1/10 the size of the parental plasmid. Our molecular manipulations have enabled us to focus upon the specific LT structural gene(s) and the controlling elements important for its expression. We have already initiated studies using the minicell system to detect the proteins encoded in this cloned LT fragment. These studies have been aided by our

recent isolation of deletions in EWD030 which have lost the capacity to synthesize LT or which now produce a nontoxic protein (data not shown). We hope these studies will yield valuable information not only on the precise molecular nature of LT but also on the factors controlling toxin biosynthesis. Of course, the fact that cells harboring ESF3003 produce a several-fold-higher level of toxin due to a gene dosage effect should be advantageous for large-scale toxin isolation and characterization.

Enterotoxigenic *E. coli* have been isolated from calves, lambs, piglets, and humans, and the genetic information for the synthesis of both ST and LT has been found on the same plasmid, as well as occurring independently. Because the precise characterization of *E. coli* enterotoxins has been elusive, the question of the relationships among the LT structural genes from different animal sources and plasmids has remained unanswered. Whether LT from LT plasmids of human origin are related to LT plasmids of either bovine or porcine origin is unknown. By using the cloned LT fragment as a probe in DNA-DNA hybridization experiments, we hope to investigate the questions of relatedness between LT structural genes and also other questions concerning the epidemiology of toxigenic *E. coli*. At a more practical level, we hope that these cloned DNA fragments encoding LT synthesis will eventually provide one of the means for specific immunoprophylaxis for toxigenic diarrheal disease.

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We thank James J. Champoux and Betty L. McConaughy for their expert advice on matters of tissue culture. We also thank Carlton Gyles and H. William Smith for strains.

LITERATURE CITED

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